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Identification and Transcriptional Analysis of a 3′-Coterminal Gene Cluster Containing UL1, UL2, UL3, and UL3.5 Open Reading Frames of Bovine Herpesvirus-1[1]

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We have identified and sequenced 3113 nucleotides located at the right end of the *Hin*dIII L fragment of the bovine herpesvirus-1 genome from map units 0.712 to 0.734. Analysis of the sequence identified four open reading frames (ORFs) which are designated UL1, UL2, UL3, and UL3.5 based on their homology with proteins of herpes simplex virus-1 (HSV-1), pseudorabies virus (PRV), equine herpesvirus-1, and varicella–zoster virus. The UL1 ORF of 158 amino acids exhibits limited homology with UL1 (glycoprotein gL) of HSV-1 (27%) and PRV (21%). The UL2 ORF of 204 amino acids shows significant homology to UL2 (uracil–DNA glycosylase) of HSV-1 (68%) and PRV (75%). The UL3 ORF of 204 amino acids shows significant homology to UL3 (nuclear phosphoprotein) of HSV-1 (62%) and PRV (53%). The UL3.5 ORF of 126 amino acids shows limited homology to the UL3.5 ORF of PRV (31%). The homolog of this gene is absent in HSV-1. Nucleotide sequence analyses also revealed potential TATA boxes located upstream of each ORF. However, only one polyadenylation signal was detected downstream of the UL3.5 ORF. Northern (RNA) blot analyses revealed four transcripts of 2.4, 1.9, 1.3, and 0.7 kb, which are transcribed in the same direction and are 3′-coterminal transcripts. These mRNAs appear to yield proteins encoded by UL1 (2.4 kb), UL2 (1.9 kb), UL3 (1.3 kb), and UL3.5 (0.7 kb) ORFs.

Bovine herpesvirus type 1 (BHV-1), a member of the subfamily Alphaherpesvirinae (Roizman *et al.*, 1982), genus *Varicellovirus* (Brown, 1989), is an important pathogen of cattle which causes severe respiratory infections including shipping fever (Yates, 1982). The BHV-1 genome is a linear double-stranded DNA molecule with a length of 135–140 kb (Mayfield *et al.*, 1983; Wyler *et al.*, 1989). The genome can be divided into a unique long region (U_L [105 kb]) and a short region which are covalently linked to each other (Mayfield *et al.*, 1983; Wyler *et al.*, 1989). The unique short region (11 kb) is flanked by two inverted repeats (12 kb each) which enable the viral genome to exist in two isomeric structures (Mayfield *et al.*, 1983).

The herpes simplex virus-1 (HSV-1) genome has been shown to contain at least 72 genes (McGeoch *et al.*, 1988), many of which have been characterized at the protein level. The genome of BHV-1 has not been sequenced completely; however, Northern blot analysis has identified 54 to 59 BHV-1-specific transcripts in a productive BHV-1 infection (Seal *et al.*, 1991; Wirth *et al.*, 1989). This compares well with 33 structural proteins and 15 nonstructural proteins reported to be encoded by the BHV-1 genome (Bolton *et al.*, 1983; Misra *et al.*, 1981). Although the tentative number of BHV-1-specific transcripts and proteins is known, definitive gene assignments are available for only a few proteins (Tikoo *et al.*, 1995).

In this study, we report the DNA sequence, gene arrangement, and amino acid sequence of a group of four colinear open reading frames (ORFs) of BHV-1, starting from the right end of the *Hin*dIII L fragment of the BHV-1 Cooper isolate (Mayfield *et al.*, 1983), between map units 0.734 and 0.712. Based on positional and sequence similarities between BHV-1 and other members of alphaherpesviruses (Davison and Scott, 1986; Dean and Cheung, 1993; McGeoch *et al.*, 1988), we named the identified BHV-1 ORFs as UL1, UL2, UL3, and UL3.5. Northern blot analysis helped to map the UL1, UL2, UL3, and UL3.5 transcripts and revealed complex overlapping transcripts having a similar transcription termination sequence.

MATERIALS AND METHODS

Cells and virus

The Cooper isolate of BHV-1 was grown in Madin–Darby bovine kidney (MDBK) cells cultivated in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Gibco–BRL, Burlington, Ontario, Canada). BHV-1 was assayed by plaque titration as described previously (Rouse and Babiuk, 1978).

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Cloning and DNA sequencing

The construction and restriction mapping of a pBR322-based plasmid library, containing fragments of the BHV-1 Cooper strain genome have been described in detail (Mayfield *et al.*, 1983). The *Hin*dIII L fragment cloned into pBR322 (pSD72; Mayfield *et al.*, 1983) was digested with appropriate restriction enzymes and 2.2-kb (*Hin*dIII–*Nru*I) and 1.8-kb (*Sac*I–*Sac*I) fragments (Fig. 1B) were cloned into pTZ18R and pTZ19R (Pharmacia Biotech., Baied'Urfe, Quebec, Canada). A library of nested deletions was generated using a double-stranded nested deletion kit (Pharmacia Biotech.). Single-stranded copies of cloned inserts were generated using M13KO7 helper phage. Sequencing of single-stranded DNA was performed by the dideoxy chain termination method using a T_7 sequencing kit (Pharmacia Biotech.). The analog 7-deaza-2′-deoxy-GTP was substituted for dGTP in sequencing reactions to minimize band compressions resulting from the high G+C content (72%) of BHV-1 DNA (Plummer *et al.*, 1969). Both DNA strands were sequenced for accuracy. Sequencing products were labeled with [^{35}S]dATP (Amersham, Oakville, Ontario, Canada) and visualized by autoradiography after electrophoresis. The DNA sequence was analyzed using IBI/Pustell DNA/Protein Sequence Analysis software (International Biotechnologies, Inc., New Haven, CT) and PC/Gene software (IntelliGenetics).

RNA isolation

Total cellular RNA was isolated from infected (multiplicity of infection = 10) or mock-infected cells at 2-hr intervals from 2 to 10 hr postinfection by the guanidinium method (Chomczynski and Sacchi, 1987). Briefly, BHV-1-infected and mock-infected MDBK cells were washed three times with 0.1 *M* ice-cold PBS and lysed with cell lysis buffer (containing equal amounts of water-saturated phenol and solution D [4 *M* guanidinium, 50 m*M* EDTA, 25 m*M* citric acid, 0.5% sarcosyl], 0.1 *M* sodium acetate, and 0.7% β-mercaptoethanol) pipetted directly on to the cell monolayer. The cell lysate was collected, mixed with chloroform–isoamyl alcohol solution (500 μl/2.5 ml of lysate), and centrifuged at 4000 rpm at 15° for 15 min. RNA in the aqueous phase was precipitated with isopropanol and pelleted by centrifugation. The RNA pellet was redissolved in solution D, precipitated with isopropanol, pelleted, and finally dissolved in DEPC-treated water. Polyadenylated RNA from BHV-1-infected and mock-infected MDBK cells was isolated with a Quick Prep Micro mRNA purification kit (Pharmacia Biotech.).

Northern (RNA) blot analysis

Equal amounts of RNA (20 μg) were run through a 1% agarose gel containing formaldehyde (Sambrook *et al.*, 1989). After electrophoresis, RNA was transferred to Zeta probe nylon membrane (Pharmacia Biotech.) with 20× *SSC (1× SSC is 0.15 M* NaCl with sodium citrate at 0.015 *M*, pH 7.0) by the method of Southern (1975). The membrane was baked for 2 hr at 80° under vacuum. The *Hin*dIII L fragment and its subclones were radiolabeled with [α-^{32}P]dCTP by nick translation (Rigby *et al.*, 1977). Oligonucleotides (27- and 30-mer) were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Sambrook *et al.*, 1989). The filters were prehybridized for 4 to 6 hr in a prehybridization buffer containing 5× SSC, 5× Denhardt's (1× Denhardt's solution is 0.2% each bovine serum albumin, Ficoll, and polyvinylpyrollidone), 50% formamide, 50 m*M* NaH_2PO_4, 0.1% SDS, 1% glycine solution (combined pH of solution is 6.5). Hybridization with nick-translated and ^{32}P-end-labeled oligonucleotide probes was performed at 45° in the presence of prehybridization buffer. The blots were washed with 2× SSC containing 0.1% SDS for 15 min at room temperature twice, then at room temperature with 0.5× SSC containing 0.1% SDS for 30 min, and finally with 0.1× SSC containing 0.1% SDS at 45° for 30 min before exposing to X-Omat AR film (Eastman Kodak, Rochester, New York) at −70°.

Nucleotide sequence accession number

The nucleotide sequence data reported here have been submitted to the GenBank database and assigned Accession No. U32173.

RESULTS

Sequence analysis

We have determined the nucleotide sequence of the right-hand portion of the *Hin*dIII L fragment of the BHV-1 Cooper isolate (pSD72; Mayfield *et al.*, 1983) from 0.734 to 0.712 map units. The sequenced region contains 3113 bp and is G+C rich (71%). The nucleotide sequence is numbered from right to left with respect to the conventional BHV-1 genome map, beginning with the *Hin*dIII site at the junction of *Hin*dIII L and C fragments (Mayfield *et al.*, 1983). ORF and codon usage analysis indicated the presence of four genes oriented in the same direction. Since the prototypic U_L region of HSV-1 (McGeoch *et al.*, 1988) runs antiparallel to the U_L region of BHV-1 (Mayfield *et al.*, 1983; Tikoo *et al.*, 1995), we analyzed putative translational products of these genes for homology with proteins encoded at the left end of the HSV-1 U_L region (McGeoch *et al.*, 1988). The deduced amino acid sequence of the second ORF revealed the presence of a uracil–DNA glycosylase signature sequence (Caradona and Cheng, 1980) with 68% homology to the HSV-1 UL2 product (uracil–DNA glycosylase; Mullaney *et al.*, 1989), a well-conserved gene within herpesviruses. Since amino acid sequence analysis of ORFs 1 and 3 also showed significant homology to the HSV-1 UL1 and UL3 gene products (McGeoch *et al.*, 1988), respectively, we designated these genes BHV-1 UL1, UL2, UL3, and UL3.5. The location and the predicted amino acid sequences of these ORFs are depicted in Figs. 1 and 2.

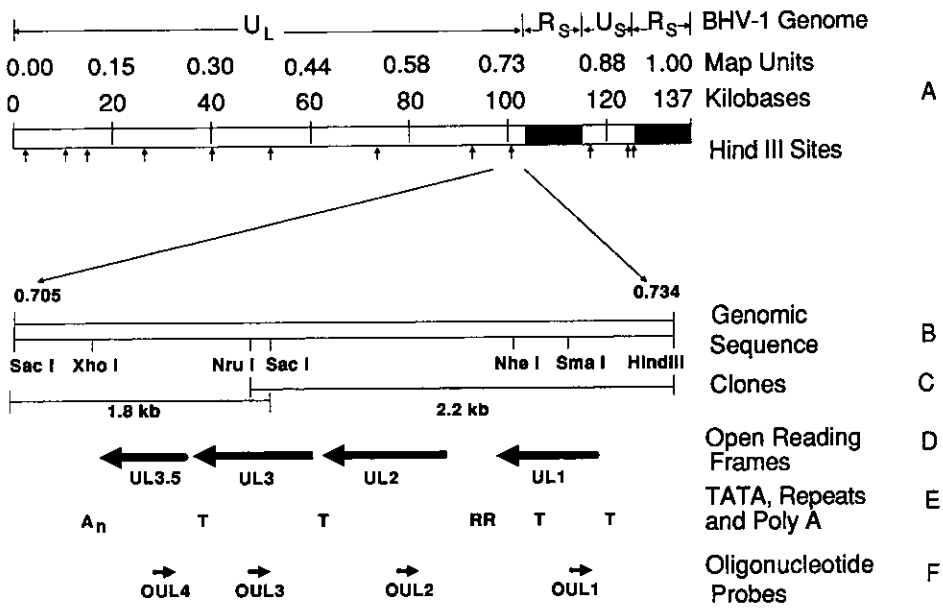

FIG. 1. Map location of the BHV-1 UL1–UL3.5 gene cluster. (A) Schematic diagram of the BHV-1 (Cooper strain) genome as adapted from Mayfield *et al.*, 1983. The genome is divided into a unique long (U_L) and a unique short (U_S) segment flanked by inverted repeat regions (R_S). Locations of *Hin*dIII restriction endonuclease cleavage sites are indicated. (B) Expanded portion of 0.734 to 0.705 map units of *Hin*dIII L fragment. (C) Enlarged portion of *Hin*dIII–*Nru*I and *Sac*I–*Sac*I overlapping clones. (D) Locations of open reading frames UL1, UL2, UL3, and UL3.5. Arrows indicate direction of transcription. (E) Locations of TATA boxes (T), repeats (RR), and polyadenylation site (A_n). (F) Locations and orientations of oligonucleotide probes.

UL1

The UL1 ORF starts at nucleotide 363 and ends at nucleotide 836 encoding a protein of 158 amino acids (Fig. 2) with a predicted molecular mass of 17 kDa and an isoelectric point of 7.2. It contains an N-terminal signal sequence of 25 amino acids as the length, relative hydrophobicity, and consensus cleavage site is characteristic of eukaryotic signal sequences (Fig. 2; von Heijne, 1986). Cleavage of the signal sequence would be predicted to occur after the alanine residue at position 25, such that alanine would occupy both positions at −3 and −1 and would conform to the strict requirements for specific amino acids at these key positions (von Heijne, 1986). There is no consensus sequence for the addition of N-linked oligosaccharides (Kornfeld and Kornfeld, 1985); however, there are several serine and threonine residues which could accept O-linked oligosaccharides. The location of the UL1 gene in BHV-1 is equivalent in genome location to pseudorabies virus (PRV) UL1 (Dean and Cheung, 1993), HSV-1 and HSV-2 UL1 (McGeoch *et al.*, 1988, 1991), varicella–zoster virus (VZV) ORF 60 (Davison and Scott, 1986), equine herpesvirus-1 (EHV-1) ORF 63 (Telford *et al.*, 1992), and Marek's disease virus (MDV) UL1 (Yoshida *et al.*, 1994) genes. Since the gene arrangement appears to be conserved in alphaherpesviruses (Dean and Cheung, 1993), including BHV-1 (Fig. 1), the BHV-1 UL1 is expected to encode a positional gL homolog (Forghani *et al.*, 1994; Hutchinson *et al.*, 1992; Klupp *et al.*, 1994). Immediately downstream of UL1 is a region of four repeats of CCCCGG and six repeats of CTTCGGCCCCGG from nucleotide 845 to 940 (Fig. 2).

Comparison of the predicted translation product of BHV-1 UL1 with homologs of four alphaherpesviruses shows limited sequence homology (12%; Fig. 3). As shown in Fig. 3, a highly conserved region extends from amino acid 61 to 88 in BHV-1 UL1. Of the 28 residues in this region, 10 residues are conserved among all five UL1 homologs. BHV-1 UL1 has 27, 24, 33, 29, 21, and 15% homology with HSV-1 UL1 (McGeoch *et al.*, 1988), HSV-2 UL1 (McGeoch *et al.*, 1991) EHV-1 ORF 63 (Telford *et al.*, 1992), MDV UL1 (Yoshida *et al.*, 1994), PRV UL1 (Dean and Cheung, 1993), and VZV ORF 600 (Davison and Scott, 1986), respectively.

UL2

The BHV-1 UL2 ORF is located between nucleotide 1132 and nucleotide 1743 (Fig. 2). No TATA box is found between the termination codon of UL1 and the start codon of UL2; however, a potential TATA box for UL2 gene is found at nucleotide 656 (5′TATAA3′) within the UL1 gene sequence (Figs. 1 and 2). BHV-1 UL2 is predicted to encode a protein of 204 amino acids with a molecular mass of 22 kDa and an isoelectric point of 10.5. The predicted translation product contains a consensus uracil–DNA glycosylase signature sequence (5′WARRGVLLLN3′) (Caradonna and Cheng, 1980; Sancar and Sancar, 1988) from amino acids 97 to 106 (Fig. 2). BHV-1 UL2 ORF is 68% homologous with HSV-1 and HSV-2 UL2 ORFs (McGeoch *et al.*, 1988, 1991), both of which have been shown to have uracil–DNA glycosylase activity (Mullaney *et al.*, 1989; Worrad and Caradonna, 1988). The

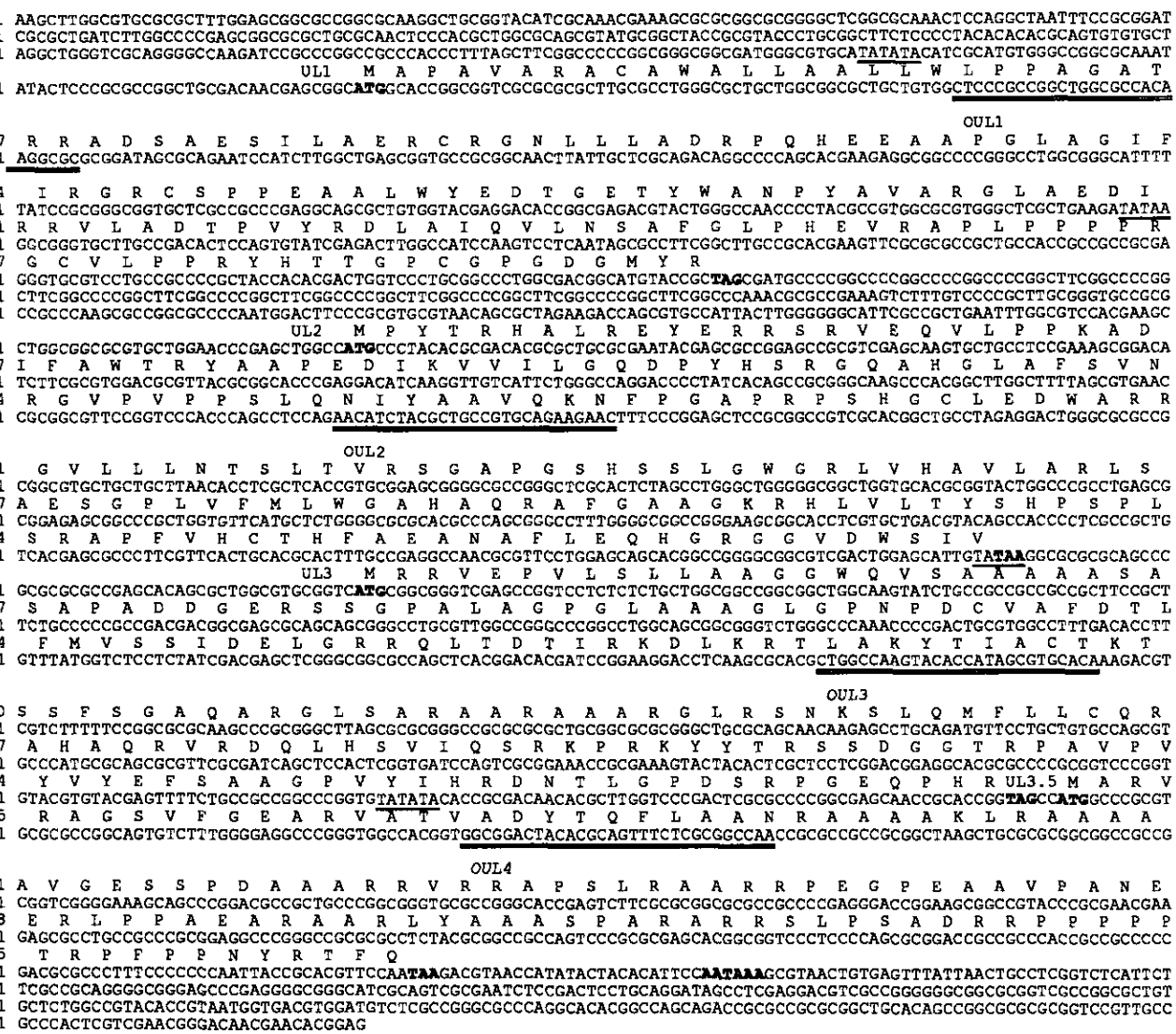

FIG. 2. DNA sequence and deduced amino acid sequence of the BHV-1 UL1–UL3.5 gene cluster. Nucleotide sequence of the 3113-bp-long, right-hand portion of the pSD72 (*Hin*dIII L fragment) of BHV-1 Cooper strain from 0.734 to 0.712 map units. Nucleotide sequence is numbered from right to left with respect to the conventional BHV-1 genome map, beginning with *Hin*dIII site at the junction of *Hin*dIII L and C fragments (Mayfield *et al.*, 1983). The deduced amino acid sequences of UL1, UL2, UL3, and UL3.5 ORFs are indicated. Locations of translation initiation codons, termination codons, and polyadenylation signal are indicated by bold letters. Putative TATA boxes are indicated by a single underline. The 27- or 30-mer oligonucleotide probes used for transcriptional analysis are complimentary to the nucleotide sequences and are indicated by a double underline.

homology of BHV-1 UL2 with PRV UL2 (Dean and Cheung, 1993), EHV-1 ORF 61 (Telford *et al.*, 1992), MDV UL2 (Yoshida *et al.*, 1994), VZV ORF 59 (Davison and Scott, 1986), Epstein–Barr virus (EBV) BKRF3 (Baer *et al.*, 1984), human cytomegalovirus (HCMV) UL114 (Chee *et al.*, 1990), and human (Olsen *et al.*, 1989) and *Escherichia coli* (Varshney *et al.*, 1988) uracil–DNA glycosylase is 75, 72, 69, 65, 57, 55, 55, and 58%, respectively. The overall homology among uracil–DNA glycosylases sequences is 21% (Fig. 4).

UL3

The BHV-1 UL3 ORF is located between nucleotide 1793 and nucleotide 2404 (Fig. 2). It encodes a protein of 204 amino acids with a molecular mass of 22 kDa and an isoelectric point of 10.8. A potential TATA box is located upstream of UL3 at nucleotide 1742 (5′TATAA3′) just before the stop codon of UL2. The UL3 gene contains two additional in-frame ATG codons at nucleotide 1985 and nucleotide 2180, which if used would yield protein products of 140 and 75 amino acids, respectively. A potential nuclear localization signal (5′RKPRK3′) (Worrad and Caradonna, 1993) is located at the C-terminus of the protein from amino acids 153 to 157 (Fig. 2). Comparison of BHV-1 UL3, HSV-1 and HSV-2 UL3 (McGeoch *et al.*, 1988, 1991), MDV UL3 (Yoshida *et al.*, 1994), EHV-1 ORF 60 (Telford *et al.*, 1992), VZV ORF 58 (Davison and Scott, 1986), and PRV UL3 (Dean and Cheung, 1993) amino acid sequences shows significant sequence homology (38%; Fig. 5). BHV-1 UL3 has 62, 58, 56, 53, 52, and 62%

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BHV-1 MAPAVARACAWA----------------------------------LLAALLWLPPA--------GATRRADSAES   34
EHV-1 MTQ-ILIGCVWQKSPYINQCTEFQPPLSFVTPERMRRMRCWARLELVYMLAWIVTTKLVKATRLDFTWGPGEPKR   74
HSV-1 MG-------------------------------------ILGW--VGLIAVGVLCVRGGLPSTEYVIRSRVAREVGD   38
HSV-2 MG-------------------------------------FVCL--FGLVVMGAWGAWGGSQATEYVLRSVIAKEVGD   38
PRV   M~RPIAPRSASPRSS----CPA--PGTWHVFPPRKSR-------GIMRMSSLCL-------NFLFPEIKAGTPAP   54
      #                                          ^** ^  ^            *  * *

BHV-1 ILAERCRGNLLLADRPQHEAAAPGL----AGIFIRGRCSPPEAALWYEDTGETYWANPYAVARGLAEDIRRVLAD  105
EHV-1 ILEASCGSGPIMKGQLFTSPNIKNLLNRTTGIMVKAHCNPPEAILWVDTPPKPVWVNPFAVVQGLAEDVTNG-NM  148
HSV-1 ILKVPCVPLPSDDLDWRYETPSAINYALIDGIFLRYHCPGLDTVLWDRHAQKAYWVNPFLFVAGFLEDLSYPAFP  113
HSV-2 ILRVPCMRTPADDVSWRYEAPSVIDYARIDGIFLRYHCPGLDTFLWDRHAQRAYLVNPFLFAAGFLEDLSHSVFP  113
PRV   KKDTPAAMSPLVAVLVFFSAALG-----VPGPGVAGNPRGLDAIFEAPVTPAPPTRHPRREELEWDDEDHPLLDL  124
      ^^  *^   ^       **          #^^*^ *^ ^^** *^   *  *^^^*#^    *  **

BHV-1 TPVYRDLAIQVLNSAFGLPHEVRAP-LPPPPR--GCVL-------------------PPRYHTT-----------  147
EHV-1 PQDFKEKLLFALDDSLSQSQSSPDEILGPPPL--GCFT-------------------GPFFLSPPKSKDIAEGL-  201
HSV-1 ANTQETETRLALYKEIRQALDSRKQAASHTPVKAGCVNFDYSRTRRCVGRQDLGPTNGTSGRTPVLPPDDEAGLQ  188
HSV-2 ADTQETTTRRALYKEIRDALGSRKQAVSHAPVRAGCVNFDYSRTRRCVGRRDLRPANTTSTWEPPVSSDDEASSQ  188
PRV   EPPVGSRCHPYIAYSLPPDMNAVTSVVVKP----------Y-----CSPPEVILWASGTAYLVNPFVAIQALAV-  183
            *    ^*  **    *^^       *^   ^^                    ^*    ^^   ^

BHV-1 ---------------------GPCGPGDGIY-----R                                        158
EHV-1 ---------------------KDSCIPA-SYYANLQKT                                      217
HSV-1 PKPLTTPPPIIATSDPTPRRDAATKSRRRRPHSRRL                                        224
HSV-2 SKPLATQPPVLALSNAPPRRVSPTRGRRRHTRLRRN                                        224
PRV   GEPLN-EAALKELGEVAVHKDS-LPPLRYNGGPPAE                                        217
        ^^                ^*  ^  ^
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FIG. 3. Amino acid homology of alphaherpesvirus UL1 homologs. Predicted amino acid sequences of UL1 homologs of HSV-1 (McGeoch *et al.*, 1988), HSV-2 (McGeoch *et al.*, 1991), EHV-1 (Telford *et al.*, 1992), and PRV (Dean and Cheung, 1993) were compared with the predicted amino acid sequence of BHV-1 UL1. Gaps (dashes) in the protein sequences have been introduced to yield maximal alignment. Perfectly conserved, well-conserved (conservative amino acid substitutions), and 3 of 5 identical residues (consensus sequence) are indicated by #, *, and ^ signs, respectively. Alignment and homology searches were performed by using the CLUSTAL program of PC/GENE software (IntelliGenetics).

homology at amino acid level with HSV-1 UL3, HSV-2 UL3, MDV UL3, PRV UL3, VZV ORF 58, and EHV-1 ORF 60, respectively.

UL3.5

The BHV-1 UL3.5 ORF starts at nucleotide 2410 and terminates at nucleotide 2787 (Fig. 2). It encodes a protein of 126 amino acids with the predicted molecular mass of 13 kDa and an isoelectric point of 12.2. A potential TATA box (5′TATATA3′) is located upstream of UL3.5 at nucleotide 2345 to 2350 within the UL3 ORF (Fig. 2). The BHV-1 UL3.5 contains many alanine (28%) and arginine (17%) residues. Comparison of the BHV-1 UL3.5 amino acid sequence with homologs in PRV (Dean and Cheung, 1993) and EHV-1 (Telford *et al.*, 1992) show limited homology (27%; Fig. 6), particularly in the N-terminal region where 33 of 50 amino acids are conserved.

The sequences upstream and downstream of the ORFs were also analysed for *cis*-acting elements (Figs. 1 and 2). Four potential TATA boxes, located at nucleotide 301 (5′TATATA3′), 656 (5′TATAA3′), 1742 (5′TATAA3′), and 2345 (5′TATATA3′) were found in the UL1 to UL3.5 gene cluster (Fig. 2). The first TATA box is upstream of the start of UL1, the second is within the UL1 coding sequence, the third overlaps with the stop codon of UL2 and is before the start codon of the UL3 gene, and the fourth is within the UL3 nucleotide sequence before the stop codon of UL3 and the start codon of the UL3.5 ORF (Fig. 2). The only polyadenylation consensus signal (5′AATAAA3′) of this gene cluster is located 3′ to the UL3.5 ORF at nucleotide 2816 (Figs. 1 and 2). The sequence 5′TTTATT3′ at nucleotide 2835 (Fig. 2) downstream of the polyadenylation signal of this gene cluster may serve as a potential polyadenylation signal for a BHV-1 UL4 gene (Vlcek *et al.*, 1995) transcribed in the opposite direction.

Transcriptional mapping of UL1 to UL3.5 ORFs

Northern blot analysis was performed to determine the transcripts originating from the UL1–UL3.5 ORFs. A 2.9-kb DNA probe (Fig. 1B; Fig. 2, nucleotide 2 to nucleotide 2928) was hybridized to total RNA from BHV-1-infected (Fig. 7, lane b) or mock-infected (Fig. 7, lane a) MDBK cells. As shown in Fig. 7 (lane b), the DNA probe hybridized to five transcripts of 3.4, 2.4, 1.9, 1.3, and 0.7 kb.

Since sequence analysis identified only one consensus polyadenylation signal downstream of ORF 3.5, we wished to determine if these transcripts are coterminal. Northern blots were probed with synthetic oligonucleotides derived from ORF 3.5. Oligonucleotide OUL4, complementary to the sequence shown in Fig. 2, hybridized to all four transcripts of 2.4, 1.9, 1.3, and 0.7 kb (Fig. 8, lane a) while oligonucleotide probes complementary to the opposite strand or derived downstream of polyadenylation site did not hybridize to any of the four transcripts (data not shown). This suggests that all four mRNA species are transcribed in the same direction, have some common sequences, and terminate at the polyadenylation site at nucleotide 2816 to 2821 (Fig. 2). Although the 1.9-kb transcript appeared early, all four transcripts were detected at 6 hr postinfection (Fig. 9).

To determine the coding potential of each transcript, Northern blots were probed with oligonucleotides predicted to hybridize to transcripts containing ORFs UL1, UL2, UL3, and UL3.5. The complementary sequences and locations of selected oligonucleo-

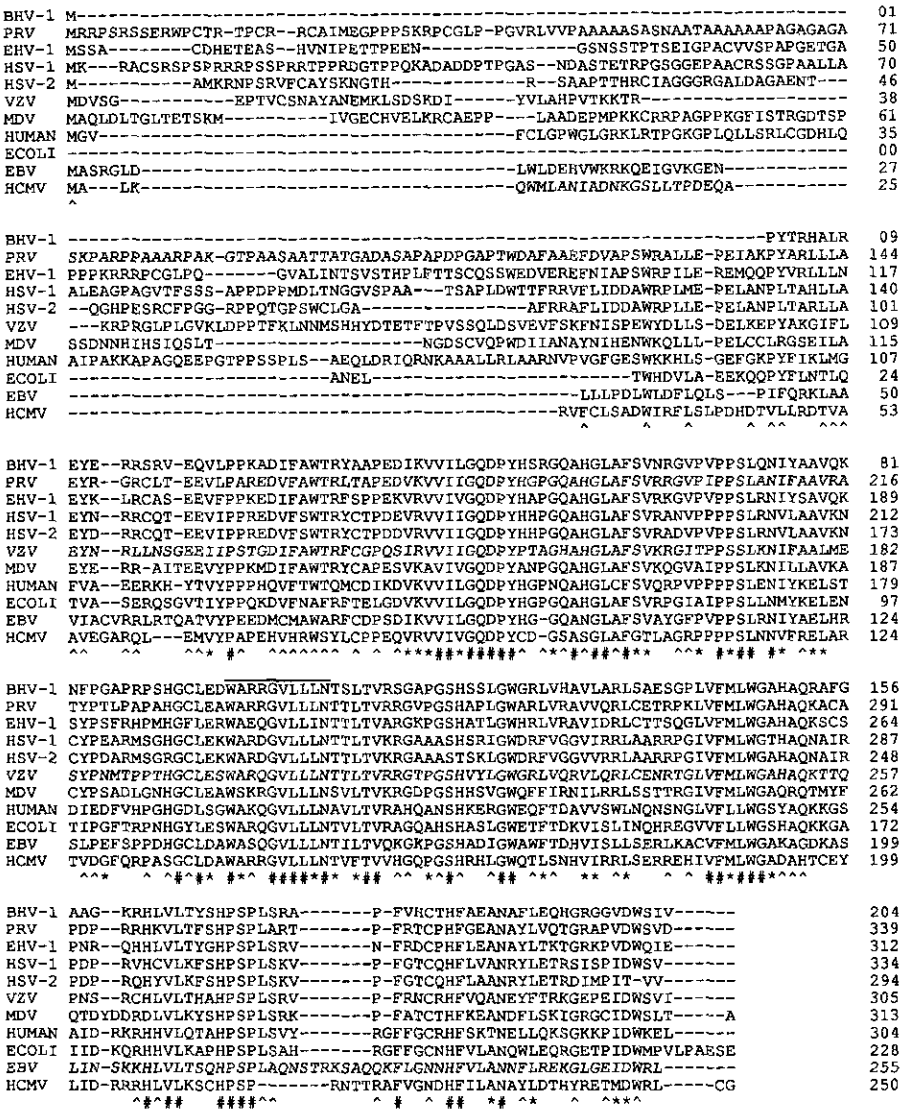

FIG. 4. Amino acid homology of BHV-1 *UL2*-like proteins. Comparison of the predicted amino acid sequence of BHV-1 *UL2* with the predicted amino acid sequences of PRV UL2 (Dean and Cheung, 1993), EHV-1 ORF 61 (Telford *et al.*, 1992), HSV-1 UL2 (McGeoch *et al.*, 1988), HSV-2 UL2 (McGeoch *et al.*, 1991), VZV ORF 59 (Davison and Scott, 1986), MDV UL2 (Yoshida *et al.*, 1994), human uracil–DNA glycosylase gene (Olsen *et al.*, 1989), *E. coli* uracil–DNA glycosylase gene (Varshney *et al.*, 1988), EBV BKRF3 (Baer *et al.*, 1984), and HCMV UL114 (Chee *et al.*, 1990). The uracil–DNA glycosylase signature sequence is overlined. Perfectly conserved, well-conserved (conservative amino acid substitutions), and consensus residues are indicated by #, *, and ^ signs, respectively.

tides are indicated in Figs. 1 and 2 and the hybridization results are shown in Fig. 8. Probe OUL1 located within the UL1 ORF hybridized to a single transcript of 2.4 kb (Fig. 8, lane b). Probe OUL2 located within the UL2 ORF hybridized to the 2.4-kb transcript and an additional transcript of 1.9 kb (Fig. 8, lane c). Probe OUL3 located within the UL3 ORF hybridized to three transcripts of 2.4, 1.9, and 1.3 kb (Fig. 8, lane d). As mentioned above, probe OUL4 hybridized to all four transcripts of 2.4, 1.9, 1.3, and 0.7 kb (Fig. 8, lane a). Probe OUL0 upstream of UL1 ORF did not hybridize to any of these transcripts, indicating that the 2.4-kb transcript contains sequences for UL1, UL2, UL3, and UL3.5; the 1.9-kb transcript contains sequences for *UL2*, *UL3*, and *UL3.5*; the 1.3-kb transcript contains sequences for *UL3* and *UL3.5*; and the 0.7-kb transcript contains only sequences for UL3.5.

DISCUSSION

Previous studies reported the sequences of the HSV-1 UL1 to UL3 gene cluster (McGeoch *et al.*, 1988) and its homologs in HSV-2 (McGeoch *et al.*, 1991), VZV (Davison and Scott, 1986), PRV (Dean and Cheung, 1993), and EHV-1 (Telford *et al.*, 1992), and of the UL3.5 gene in PRV (Dean and Cheung, 1993) and its homologs in EHV-1 (Telford *et al.*, 1992) and VZV (Davison and Scott, 1986). To search for this gene cluster in BHV-1, we took into consideration that the prototypic orientation of the U_L region in HSV-1

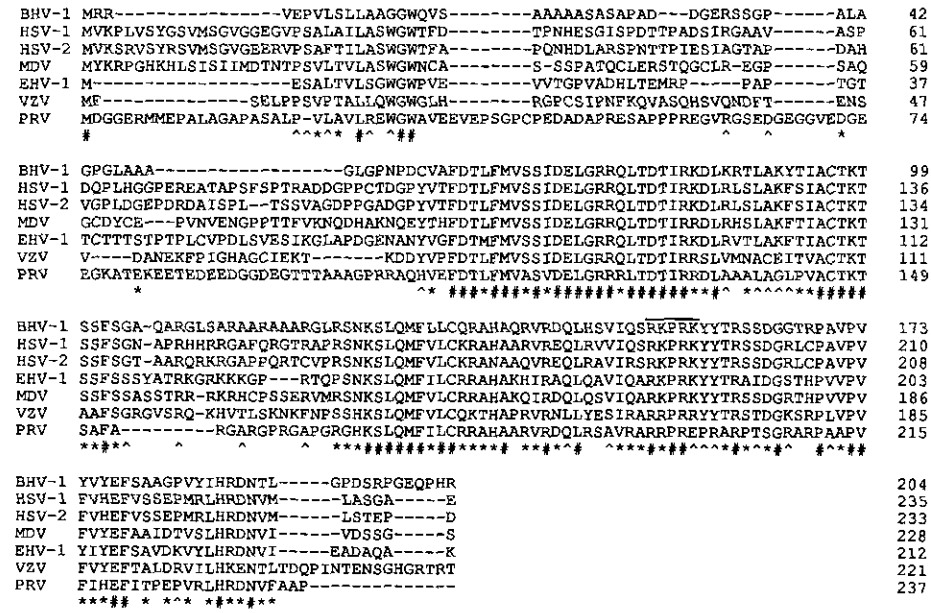

FIG. 5. Amino acid homology of BHV-1 UL3-like proteins. Alignment of deduced amino acid sequences of BHV-1 UL3 homologs with those of HSV-1 (McGeoch *et al.*, 1988), HSV-2 (McGeoch *et al.*, 1991), MDV (Yoshida *et al.*, 1994), EHV-1 (Telford *et al.*, 1992), VZV (Davison and Scott, 1986), and PRV (Dean and Cheung, 1993). The nuclear localization signal is overlined. Perfectly conserved amino acids are indicated by # and well-conserved (conservative amino acid substitutions) amino acids by *. Consensus residues are marked by ^.

(McGeoch *et al.*, 1988) is inverted relative to the orientation of BHV-1 (Mayfield *et al.*, 1983; Tikoo *et al.*, 1995), PRV (Ben-Porat *et al.*, 1983; Ben-Porat and Kaplan, 1985), EHV-1 (Telford *et al.*, 1992), and VZV (Davison and Scott, 1986). Whereas HSV-1 UL1 to UL3 gene cluster is located to the left end of the prototypic U_L region, the BHV-1 homologs are localized at the right end of the prototypic U_L region. While the gene arrangement in this region of alphaherpesvirus genomes appears to be similar, our results suggest that this gene cluster may have a different transcriptional strategy.

This region in HSV-1 contains three genes. In contrast, BHV-1, like PRV (Dean and Cheung, 1993), EHV-1 (Telford *et al.*, 1992), and VZV (Davison and Scott, 1986), contains four genes. Upstream of this gene cluster are located homologs of the immediate early ORF BICP0 in BHV-1 (Wirth *et al.*, 1992), ICP0 in HSV-1 (McGeoch *et al.*, 1988), EP0 in PRV (Cheung, 1991), ORF 61 in VZV (Moriuchi *et al.*, 1992), and ORF 63 in EHV-1 (Telford *et al.*, 1992). The UL1 ORF is conserved in genome location but not in sequence. The UL2 and UL3 ORFs are conserved both in genome location and in sequence relative to HSV-1 (McGeoch *et al.*, 1988), VZV (Davison and Scott, 1986), EHV-1 (Telford *et al.*, 1992), and PRV (Dean and Cheung, 1993). The UL3.5 ORF is conserved in genome location relative to PRV (Dean and Cheung, 1993), EHV-1 (Telford *et al.*, 1992), and VZV (Davison and Scott, 1986) with limited sequence similarity, but no counterpart, in HSV-1 (McGeoch *et al.*, 1988).

The UL1-like ORFs are preceded by a stretch of direct repeat sequences in HSV-1 (McGeoch *et al.*, 1988), PRV (Dean and Cheung, 1993), EHV-1 (Telford *et al.*, 1992), and VZV (Davison and Scott, 1986). In contrast, in BHV-1, the direct repeats of CCCCGG and CTTCGGCCCCGG are located downstream of UL1 ORF and immediately

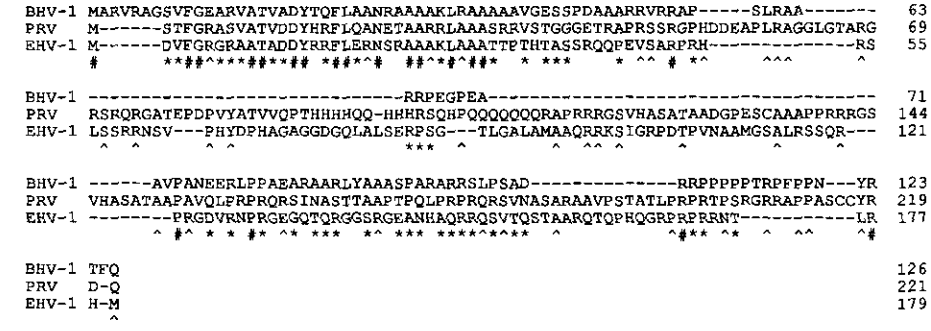

FIG. 6. Amino acid homology of BHV-1 UL3.5-like proteins. Alignment of the predicted amino acid sequence of BHV-1 UL3.5 with the predicted amino acid sequences of PRV UL3.5 (Dean and Cheung, 1993) and EHV-1 ORF 59 (Telford *et al.*, 1992) products. Perfectly conserved, well-conserved (conservative amino acid substitutions), and consensus residues are marked by #, *, and ^ signs, respectively.

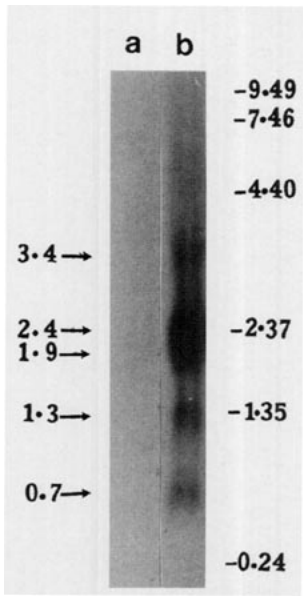

FIG. 7. Northern blot analysis of BHV-1 UL1, UL2, UL3, and UL3.5 RNA transcripts. Total RNA isolated from BHV-1-infected (lane b) or mock-infected (lane a) MDBK cells at 8 hr postinfection was hybridized to [^{32}P]dCTP-labeled 2.9-kb *Hin*dIII–*Xho*I DNA fragment. The sizes of the transcripts in kilobases are indicated on the left. The molecular sizes of marker RNAs (Gibco–BRL) in kilobases are shown on the right.

upstream of UL2 ORF. These repeats may represent part of the vestigial U_L terminal repeat sequences, as has been suggested for PRV (Dean and Cheung, 1993).

The BHV-1 UL1 ORF is colinear with the UL1 gene of HSV-1 (McGeoch *et al.*, 1988) and its homologs in PRV (Dean and Cheung, 1993), VZV (Davison and Scott, 1986), MDV (Yoshida *et al.*, 1994), EHV-1 (Telford *et al.*, 1992), HCMV (Chee *et al.*, 1990), and EBV (Baer *et al.*, 1984) but it does not show significant sequence homology with most of its counterparts. A comparison of the predicted amino acid sequence of BHV-1 UL1 with homologous

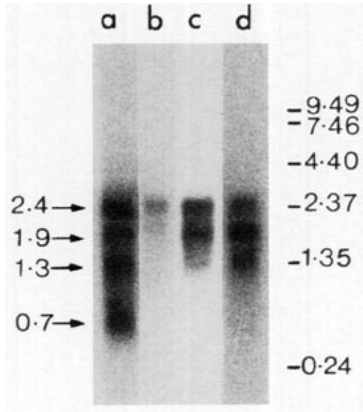

FIG. 8. Mapping of UL1 to UL3.5 transcripts by Northern blot analysis. Northern blots of polyadenylated RNA isolated from BHV-1-infected MDBK cells at 8 hr postinfection were hybridized to 27- or 30-mer γ-^{32}P-labeled OUL4 (lane a), OUL1 (lane b), OUL2 (lane c), and OUL3 (lane d) probes. The complementary sequences and locations of probes are shown in Figs. 1 and 2. The sizes of transcripts in kilobases are indicated on the left. The molecular sizes of marker RNAs (Gibco–BRL) in kilobases are shown on the right.

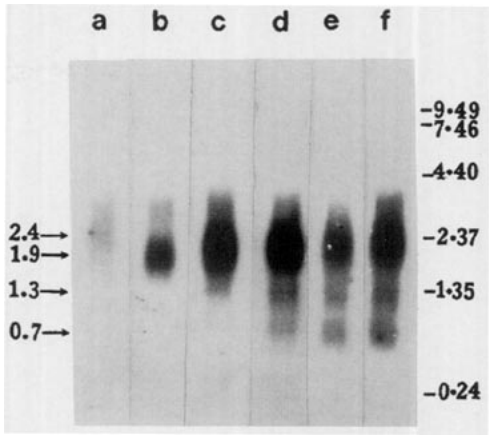

FIG. 9. Kinetics of UL1, UL2, UL3, and UL3.5 mRNA synthesis. Total RNA isolated from BHV-1-infected MDBK cells at 0 (lane a), 2 (lane b), 4 (lane c), 6 (lane d), 8 (lane e), and 10 (lane f) hr postinfection was hybridized to OUL4 oligonucleotide labeled with γ-^{32}P. The sizes of the transcripts in kilobases are indicated on the left. The molecular sizes of marker RNAs (Gibco–BRL) in kilobases are shown on the right.

proteins from other alphaherpesviruses revealed limited amino acid conservation (12% [Fig. 3]). The products of HSV-1 UL1 (Hutchinson *et al.*, 1992), PRV UL1 (Klupp *et al.*, 1994), EBV BKRF2 (Baer *et al.*, 1984), MDV UL1 (Yoshida *et al.*, 1994), HCMV UL115 (Kaye *et al.*, 1992), human herpesvirus 6 (Liu *et al.*, 1993a,b), and VZV ORF 60 (Forghani *et al.*, 1994) encode a glycoprotein named gL which acts as a chaperone molecule for the correct folding, processing, and cell surface expression of their respective gH homologs (Forghani *et al.*, 1994; Hutchinson *et al.*, 1992; Kaye *et al.*, 1992; Liu *et al.*, 1993a,b; Spaete *et al.*, 1993; Yaswen *et al.*, 1992). Despite the lack of significant sequence homology, preliminary results suggest that BHV-1 UL1 is functionally equivalent to gL, as it is required for the correct folding, processing, and cell surface expression of BHV-1 glycoprotein gH (Khattar *et al.*, unpublished data).

BHV-1 UL2, a homolog of the HSV-1 UL2 gene (McGeoch *et al.*, 1988), encodes a uracil–DNA glycosylase enzyme which acts as a DNA repair enzyme by the removal of uracil residues from DNA created from either the deamination of cytosine or the incorporation of dUMP (Caradonna and Cheng, 1980; Sancar and Sancar, 1988). The BHV-1 gene encodes a protein of 204 amino acids, which is the shortest of the reported uracil–DNA glycosylase proteins (Fig. 4; Baer *et al.*, 1984; Chee *et al.*, 1990; Davison and Scott, 1986; Dean and Cheung, 1993; McGeoch *et al.*, 1988, 1991; Mullaney *et al.*, 1989). Overall these proteins are basic in character and contain a uracil–DNA glycosylase signature sequence (Caradonna and Cheng, 1980; Sancar and Sancar, 1988). While uracil–DNA glycosylase has been shown to be dispensable for normal replication of HSV-1 in cultured cells (Mullaney *et al.*, 1989), it is required for efficient viral replication and establishment of latency in the murine nervous system (Pyles *et al.*, 1994). Its role in BHV-1 replication and pathogenesis is presently being investigated.

The BHV-1 UL3 gene encodes a protein of 204 amino acids, which shows a homology of 62% with HSV-1 UL3 (McGeoch *et al.,* 1988). Like HSV-1, BHV-1 UL3 protein contains a hydrophobic N-terminus and a potential nuclear localizing signal (RKPRK) near the C-terminal part of the protein. This nuclear localizing signal is conserved in HSV-1 (McGeoch *et al.,* 1988), HSV-2 (McGeoch *et al.,* 1991; Worrad and Caradonna, 1993), MDV (Yoshida *et al.,* 1994), VZV (Davison and Scott, 1986), and EHV-1 (Telford *et al.,* 1992). The function of the products of the UL3 family is not known. While it has been suggested that HSV-1 UL3 may code for a membrane-associated protein (McGeoch *et al.,* 1988), the HSV-2 UL3 gene has been shown to encode a nuclear localizing phosphoprotein (Worrad and Caradonna, 1993).

BHV-1 UL3.5 ORF appears to be a positional homolog of PRV UL3.5 (Dean and Cheung, 1993), EHV-1 ORF 59 (Telford *et al.,* 1992), and VZV ORF 57 (Davison and Scott, 1986) proteins as the genome locations and orientations of these ORFs are equivalent in these viruses. It has limited homology with PRV UL3 (31%), EHV-1 ORF 59 (20%), and VZV ORF 57 (30%), which is localized particularly in the N-terminal part of the proteins. The amino acid sequence of BHV-1 UL3.5 contains a basic region rich in arginine residues. This region may participate in protein–DNA interactions as has been suggested for the PRV UL3.5 gene product (Dean and Cheung, 1993).

Previous transcriptional analysis indicated six RNAs of 9.0, 5.0, 3.4, 2.8, 1.8, and 1.0 kb mapping to the *Hin*dIII L region of BHV-1 (Cooper strain), five of which were classified as late transcripts (phosphonoacetic acid sensitive) and one as an early transcript (phosphonoacetic acid resistant) (Seal *et al.,* 1991). We observed five transcripts of 3.4, 2.4, 1.9, 1.3, and 0.7 kb (mapping to 2.9 kb *Hin*dIII–*Xho*I region), four of which appear to encode the UL1 to UL3.5 gene cluster. Only one consensus polyadenylation signal was found downstream of the UL3.5 gene, which suggested that these four transcripts may overlap. Northern blot analysis confirmed that these four transcripts of 2.4 (UL1), 1.9 (UL2), 1.3 (UL3), and 0.7 (UL3.5) kb overlap and are 3′ coterminal. In addition, these results indicated that the larger transcripts contain multiple ORFs of the gene cluster. Since internal initiation of translation (Pelletier and Sonenberg, 1988) is not common in alphaherpesviruses, it is likely that only the first ORF in each transcript is translated, as UL1, UL2, UL3, and UL3.5 ORFs lie in different reading frames. A similar situation also occurs in PRV as there is only one polyadenylation signal downstream of the UL3.5 gene (Dean and Cheung, 1993) and the UL1, UL2, UL3, and UL3.5 mRNAs constitute a family of overlapping transcripts that share a 3′-terminus (Dean and Cheung, 1993). In contrast, in HSV-1, UL2 and UL3 are followed by separate polyadenylation signals downstream of their termination codons (McGeoch *et al.,* 1988) with several 3′-coterminal mRNAs being described (Worrad and Caradonna, 1988).

In addition, while in PRV (Dean and Cheung, 1993) only UL1 and UL3 of the UL1 to UL3.5 gene cluster are preceded by a separate A+T-rich eukaryotic promoter-like element, in BHV-1, like HSV-1 (McGeoch *et al.,* 1988) and HSV-2 (McGeoch *et al.,* 1991), each gene of the UL1 to UL3.5 gene cluster is preceded by a TATA box. This may suggest that each gene is transcribed from its own TATA box. Alternatively, all four genes may be transcribed from a single TATA box. Since all four transcripts are not of the same kinetic class (Seal *et al.,* 1991), the use of a single promoter for all four genes is unlikely.

In summary, we have identified a 3′-coterminal gene cluster of four genes in the BHV-1 genome named UL1, UL2, UL3, and UL3.5 which are predicted to encode glycoprotein gL, uracil–DNA glycosylase, nuclear targeting protein, and a protein of unknown function, respectively. To study the role of these genes in viral replication, we are currently making and analyzing BHV-1 mutants deficient in each of these genes.

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